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S(+)MDMA and 5-HT Evoke Dissimilar Ionic Currents in Human Serotonin Transporter-Expressing Cells
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Although monoamine transporters translocate substrates (neurotransmitters) across the plasma membrane, a second less studied function is their ion channel-like activity which is responsible for the passive permeation of ions through the membrane. These currents are inward at resting membrane potential and would result in cell depolarization, which may enhance presynaptic excitability, and which would help explain the elevated release of neurotransmitter associated by psychostimulants. Here we compare the ionic currents evoked by serotonin (5-HT) and S(+)-3,4-methylenedioxy-N-methylamphetamine (S(+)MDMA, ecstasy) in mammalian cells expressing the human serotonin transporter (hSERT). 5-HT as well as S(+)MDMA induce inward currents in hSERT-expressing cells. Although the conductances are similar, the S(+)MDMA induced-current reversal potential is strongly shifted towards the Na^+ equilibrium potential, indicating a bigger contribution of Na^+ to the overall current compared to 5-HT current. To measure changes in Na^+ permeability induced by these compounds, the cells were pre-loaded with the Na^+ sensor Asante NaTRIUM GREEN 2 and changes in intracellular Na^+ concentration ($[Na^+]i$) were monitored fluorometrically. Dose-response experiments show that 5-HT and S(+)MDMA have similar potency (EC50 ~200 nM) in elevating the $[Na^+]i$; however, S(+)MDMA increases $[Na^+]i$ at least 3 times with respect to 5-HT. In addition, the reconstitution of hSERT on a lipid bilayer in the presence of a Na^+ gradient showed channel-like activity after the application of 5-HT or S(+)MDMA in the trans side. Taken together these results strongly suggest that S(+)MDMA-induced current is different in composition than the 5-HT current, and moreover it must be strongly depolarizing due to its higher Na^+ content.
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Is the Second Sodium Pump Electrogenic?
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Transepithelial sodium transport is a process that involves Na^+ entry across the apical membrane of the epithelial cell following its electrochemical gradient and its active extrusion at the basolateral plasma membrane by two sodium pumps: The Na/K-pump, which depends on K^+, is inhibited by ouabain and insensitive to furosemide; and the Second Sodium pump, which is K^+-independent, insensitive to ouabain but inhibited by furosemide (J. Gen. Physiol. 51:303s-314s, 1968; Pflügers Archiv Eur J Physiol 316:1-25, 1970; Biochim Biophys Acta 394:281-292, 1975). Both transport mechanisms are associated with two ATPases present in the basolateral plasma membrane of the epithelial cells with similar functional characteristics, the Na^+/K^+-ATPase and the Na^+-ATPase, respectively (Biochim Biophys Acta 1808:1684-1700, 2011; Pflügers Archiv Eur J Physio. 316:1-25, 2012). The Na^+/K^+-pump is electrogenic; it exchanges 3 internal Na^+ by 2 external K^+ producing a net charge movement. The Second pump transports Na^+ with Cl^- and water. It has been suggested that this pump could be electrogenic. The Na^+ charge transfer would induce Cl^- movement and osmotic movement of water following the movement of both Na^+ and Cl^- across the membrane. We have evaluated electrical parameters of the basolateral plasma membranes of MDCK cells cultured on Transwell in Ussing chambers, using amphotericin B as apical permeabilizing agent. Our results confirm that the Na^+/K^+-pump is electrogenic and that the Second sodium pump is electroneutral, coupling the active Na^+ transport to a specific Cl^- movement through a particular conductive pathway.

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β-Exodomain Role in Na/K-ATPase α-β Subunit Interactions
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We disrupted the three conserved disulfide bonds in *Xenopus* β3 (C127−C144, C154-C170, C191-C248), one at a time, using Cys to Ala/Ser mutations and coexpression with relatively ouabain-resistant (C113Y) *Xenopus* α1 in oocytes. We assessed function with two-microelectrode voltage clamp in 1μM ouabain to silence endogenous Na/K pumps. Single C127S/A or C154S, or double C127S+C154S β3 mutants yielded steady Na/K pump currents (saturating [Ko]) and transient Na charge movements (without Ko) like those of C113Y α1 coexpressed with wild-type β3, suggesting that neither assembly, trafficking, nor Na/K pump function require intact first and/or second disulfide bonds. In contrast, steady and transient pump currents were both ≥10 fold smaller in Na/K pumps with disrupted third disulfide bond after C113Y α1 coexpression with C191A/S β3. Similarly, in outside-out excised patches, palytoxin-induced pump-channel currents were ≥10 fold smaller for C113Y α1 Na/K pumps coexpressed with β3 C191S than with wild-type β3. Because in uninjected oocytes or oocytes expressing C113Y α1 alone, neither steady nor transient pump current could be detected, and palytoxin elicited no measurable current in outside-out patches, the third disulfide bond appears necessary for efficient Na/K-ATPase assembly and trafficking to the cell membrane but is not essential for assembled pump function. We disrupted salt bridges linking the αTM7-TM8 loop and β-exodomain in Na/K-ATPase structures (α1 E901-β3 K219, α1 E911-β3 R262), by coexpressing E901R(C113Y) or E911R(C113Y) α1 with wild-type β3. In both mutants, steady Na/K pump currents (at saturating [Ko]) and transient Na charge movements were like wild type; but the E901R α1 mutation selectively enhanced apparent Ko affinity ~2 fold. These results suggest that some β-exodomain structural alterations can be tolerated; but α-β subunit interface interactions closely regulate Ko binding/occlusion steps, though not the slow step that occludes/deoccludes the third Na ion. [HL36783]

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Protein Translocation at the Single Molecule Level
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All proteins are synthesized in the cytoplasm. However, for many of them the cytoplasm is not their final destination. Instead, they need to be translocated across or into the plasma membrane. In bacteria, this process is mediated by the membrane-embedded protein-conducting SecYEG channel. This channel can either associate with the ribosome (co-translational translocation) or with a cytosolic motor protein, the SecA ATPase (post-tranlational translocation), with each the ability to provide the driving force for the translocation process across the membrane.
Even though the SecYEG system has been intensively studied, many aspects of protein translocation remain elusive. Here, we study translocation by single-molecule fluorescence imaging. We reconstitute the bacterial SecYEG into phospholipid bilayer nanodiscs and immobilize these on a functionalized glass-surface. By monitoring the interactions of the components of the Sec translocon at resolution of individual molecules we aim to provide a means for better understanding the journey of proteins into or across the membrane.

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Studies of the Conformational Dynamics of Ligand and Nucleotide Bound P-Glycoprotein
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A major factor contributing to the therapeutic effectiveness of a drug is whether it will be a substrate for efflux transporter proteins such as P-glycoprotein (P-gp). P-gp is an ATP-binding cassette transporter that is able to expel a remarkable range of therapeutic drugs from their target cells. Despite this, surprisingly little is known about the mechanism with which it exports drugs. Following drug binding within P-gp's transmembrane domain (TMD) binding cavity, export to the cell exterior is believed to be driven by ATP binding and/or hydrolysis at the cytoplasmic nucleotide binding domains (NBDs). We have been using atomistic molecular dynamics simulation to study the interactions of experimentally characterized ligands with the binding cavity of P-gp and their influence on P-gp NBD dynamics.
Available structures of P-gp display large separations of their NBDs. We observed tighter association of the NBDs in our simulations even in the absence of nucleotide. The degree of association and ATP binding site conformations were dependent on whether substrate or inhibitor was bound in the TMD binding cavity. In addition, conformational changes in the binding cavity coupled with ligand dynamics allowed formation of protein-ligand contacts that agreed with previous mutational studies but could not be predicted from just the crystal structure. Finally we explored the effects of ATP binding and hydrolysis to better understand how this process is coupled to substrate transport.

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The IC50 for Inhibition of Digoxin Transport Across Confluent Cell Monolayers of P-gp Expressing Cell Lines is Often a Function of Inhibitor Binding to Both P-gp and an Unidentified Basolateral Digoxin Uptake Transporter
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Previously, we reported that the P-gp substrate digoxin requires basolateral and apical uptake transporter(s) to achieve the observed efflux kinetics across MDCKII-MDR1 (Netherlands Cancer Institute) confluent cell monolayers.

We extend this kinetic analysis to include other cell lines, including MDCKII-MDR1 (National Institute of Health) and Caco-2. Digoxin transport across confluent cell monolayers was measured in the absence and presence of several P-gp substrates and/or inhibitors. Our kinetic analysis showed that digoxin transport in these other cell lines typically requires a basolateral digoxin uptake transporter. The B>A transport inhibition curve of digoxin was a conflation of inhibitor binding to both P-gp and to this basolateral digoxin uptake transporter. If a new compound is an inhibitor of digoxin transport across any of these cell-lines, the IC50 could be due to binding to P-gp or to the basolateral digoxin uptake transporter, or both. Finally, our analysis allowed us to estimate the efflux active P-gp surface density on the cell lines, which is the fraction of P-gp in the plasma membrane which successfully effluxes substrate into the apical chamber, which depends upon microvilli morphology.

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Low Doses of a Combination of Curcumin with Ouabain or Gramicidin Selectively Kill Cancer Cells that Express the Multidrug Resistance-Linked ABCG2 Transporter

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The work presented here introduces a strategy to kill selectively multidrug resistant (MDR) cancer cells that express the ABCG2 transporter (also called breast cancer resistance protein, BCRP). The approach is based on specific stimulation of ATP hydrolysis by ABCG2 transporters with sub-toxic doses of curcumin combined with stimulation of ATP hydrolysis by the Na^+ K^+ ATPase with sub-toxic doses of ouabain or gramicidin A. The resulting over-consumption of ATP by both pathways inhibits the efflux activity of ABCG2 transporters and leads to depletion of intracellular ATP levels below the viability threshold. In contrast, cells without the ABCG2 transporter lack one pathway and maintain viability at basal ATP levels. Similar results were obtained with a clinically relevant human breast adenocarcinoma cell line that expresses ABCG2 transporters (MCF-7/FLV1) and its parental cell line (MCF-7). This strategy, which is based on compounds that are currently in clinical use, exploits the overexpression of ABCG2 transporters in cancer cells by accelerating their ATP hydrolysis rate to kill selectively those cells which are particularly resistant to conventional chemotherapy. This work introduces a novel strategy to exploit collateral sensitivity (CS) with a combination of two compounds that, individually, do not exert CS. It also demostrates that the mechanism for CS is necrotic cell death induced by depletion of ATP below 50% of basal intracellular levels. Since ABCG2 transporters are a putative marker for cancer stem cells (CSCs), this approach may allow targeting CSCs.

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Gramicidin Currents Report P-Glycoprotein in Patch Clamp Recordings

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P-glycoprotein (P-gp), a member of the ATP binding cassette (ABC) transporter family, actively excludes chemotherapeutics leading to reduced efficacy of these agents, and the phenomenon of multidrug resistance (MDR) in cancer cells. Thus, development of functional assays to better characterize P-gp and minimize MDR is important for cancer therapy. Previous report demonstrated that efflux of gramicidin D, a pore-forming antibiotic peptide, by P-gp reduced pore formation, resulting in decreased cellular accumulation of $^{86}Rb+$. This result suggested that gramicidin D is a substrate of P-gp [1]. This work presented here establishes a novel approach to monitor the efflux activity of P-gp directly and in real time. This approach takes advantage of the single molecule sensitivity of patch clamp techniques to measure currents through pores of the ion channel forming peptide, gramicidin A (gA), which is also a substrate of P-gp. Therefore, changes in whole cell gA currents directly report the efflux of gA by P-gp. For instance, parental cells that do not express P-gp show whole cell gA currents in a dose dependent manner, whereas resistant cells that over-express P-gp show significantly reduced gA currents. P-gp inhibitors restore gA currents to the level comparable to that in parental cells, which provides the ability to screen for competitive inhibitor of P-gp. In addition, this assay provides easy access to extracellular and intracellular solution, and direct control of cell membrane potential and intracellular concentration of molecules such as ATP and Ca^{2+}. Results demonstrate that membrane depolarization enhances the activity of P-gp while hyperpolarization reduces P-gp activity.

Reference:
1.Assaraf, Y.G. and M.J. Borgnia, Probing the interaction of the multidrug-resistance phenotype with the polypeptide ionophore gramicidin D via functional channel formation. Eur J Biochem, 1994. 222(3): p. 813-24.

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Regulation of NBCe1-B by IRBIT, PIP2 and the WNK/SPAK Kinases

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The electrogenic Na^+-HCO_3^- co-transporter NBCe1-B plays a crucial role in pH_{in} regulation and epithelial HCO_3^- secretion by mediating basolateral HCO_3^- entry, when the luminal HCO_3^- exit mediated by CFTR and members of the SLC26 transporters.

The regulation of NBCe1-B is poorly understood. IRBIT, (inositol 1,4,5-trisphosphate receptor binding protein released with IP_3), binds to N terminus of NBCe1-B to markedly increase its activity, while the WNK/SPAK pathway setting the resting state by reducing surface expression of NBCe1-B. NBCe1-B may also be regulated by PIP2 interacting with highly charge module in the N terminus. The site and mechanisms for the regulation by IRBIT, PIP2, and SPAK and the relationship between them are not known.

We identified a positive clustered module in NBCe1-B and three arginines within required for regulation by IRBIT and PIP2. The effect of IRBIT and PIP2 are not additive but complementary. The module contains two critical phosphorylation sites. The constitutively cAMP phosphorylated Thr49 is required for all form of NBCe1-B regulations, while Ser65 mediates the function of SPAK. The module is conserved in most NBC superfamily and have the same roles in NBCn1-A. These findings suggest the sites of regulation on NBCe1-B by IRBIT/PIP2 and SPAK and provide a molecular mechanism by which these regulatory factors converge to coordinate epithelia HCO_3^- secretion.

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Structural Mechanism of Action of Binding Protein Independent Mutant MalG511 of Escherichia Coli Maltose Transporter

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ATP-binding cassette (ABC) transporters couple the uphill transport of substances to the hydrolysis of ATP. Mutations in the *E. coli* maltose ABC transporter have been isolated that allow transport in the absence of periplasmic maltose binding protein (MBP), a protein that delivers maltose to the transporter with high affinity and stimulates the ATPase activity of the transporter. One such mutant, MalG511, shows an interesting biphasic behavior with low levels of MBP stimulating transport activity while higher levels (>50μM) inhibit transport activity. Remarkably, the rescuing effect of MBP suppressor mutants isolated at high, inhibitory MBP concentrations provided insight into regulatory mechanisms in the ABC transporter superfamily. Site directed spin labeling EPR spectroscopy was used to follow the catalytic cycle of MalG511 without any binding protein, with MBP and with suppressor MBP by attaching spin label at two positions in the MalK ATPase subunit (V16 and R129) that can be used to detect the closure of nucleotide-binding interface. MalG511 responded differently to the addition of ligands, including nucleotide and MBP, than wild type transporter. Notably, MalG511 can sample all conformational states (open, semi-open and closed interface) in the absence of MBP, as expected for a MBP-independent transporter. However, the closed state could be stabilized only in the presence of a transition state analogue (vanadate) suggesting that the mutations in MalG511 altered the conformational equilibrium of the transporter, favoring a semi-open over a closed conformation, even in the presence of ATP, plus or minus MBP. The addition of MBP, but not suppressor MBP, also resulted in a failure of the transporter to return to the fully open state at the end of a catalytic cycle, suggesting a mechanism for inhibition of transport at high MBP concentration.

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Conserved Residues Serine 225 and Methionine 229 Attenuate the Sodium Transport Responses in the Sodium-Coupled Neutral Amino Acid Transporter, SNAT2

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Sodium dependent neutral amino acid transporter (SNAT) 2, a member of the solute carrier (SLC) 38 family, system A, mediates the coupled transport of sodium and small neutral amino acids across a cell's membrane. There has been proposed structural homology between SNAT2 and the sodium/betaine symporter, BetP. We mutated two conserved amino acids that have been shown